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ORIGINAL ARTICLE

Tumour Suppressor Neuron Navigator 3 and Matrix Metalloproteinase 14 are Co-expressed in Most Melanomas but Downregulated in Thick Tumours

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Melanoma is a highly metastatic tumour originating from neural crest-derived melanocytes. The aim of this study was to analyse the expression of neuron navigator 3 (NAV3) in relation to membrane type-1 matrix metalloproteinase MMP14, a major regulator of invasion, in 40 primary melanomas, 15 benign naevi and 2 melanoma cell lines. NAV3 copy number changes were found in 18/27 (67%) primary melanomas, so that deletions dominated (16/27 of samples, 59%). NAV3 protein was found to be localized at the leading edge of migrating melanoma cells in vitro. Silencing of NAV3 reduced both melanoma cell migration in 2-dimensional conditions, as well as sprouting in 3-dimensional collagen I. NAV3 protein expression correlated with MMP14 in 26/37 (70%) primary melanomas. NAV3 and MMP14 were co-expressed in all tumours with Breslow thickness < 1 mm, in 11/23 of mid-thickness tumours (1–5 mm), but in only 1/6 samples of thick (> 5 mm) melanomas. Altogether, NAV3 number changes are frequent in melanomas, and NAV3 and MMP14, while expressed in all thin melanomas, are often downregulated in thicker tumours, suggesting that the lack of both NAV3 and MMP14 favours melanoma progression.

Key words: melanoma; naevus; NAV3; MMP14; copy number change.

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SIGNIFICANCE

Melanoma is the most aggressive skin cancer, and its incidence is increasing. It is important to understand which genes and proteins are involved in the malignant transformation and progression of melanoma. This study found that tumour suppressor NAV3 was deleted in the majority of melanomas, and that NAV3 protein was expressed in all the thin melanomas, but was downregulated in thick tumours, suggesting that it plays a role in melanoma progression.

Melanoma is one of the most feared cancers, since it is very therapy-resistant, and metastases can form even a decade after clinical remission. The incidence of melanoma is increasing worldwide, and the median age of patients is approximately 57 years, which is 10 years younger than that of all cancer patients (1). The prognosis of melanoma depends on its Breslow thickness and stage, as 5-year survival for stage I melanoma (localized) is 99% and for stage IV melanoma (with distant metastases) is 20% (2). Even though new treatments, such as immune checkpoint inhibitors and BRAF inhibitors, have markedly improved treatment results in patients with advanced melanoma, there is a lack of biomarkers that could predict which melanomas require sentinel lymph node biopsy and more aggressive treatment or predict WKHHIÀFDFLRQHȤWUHDWPHQWV

Evolutionally conserved genes involved in cell proliferation and motility are likely to contribute to melanoma cell invasion and spreading. One such gene, neuron navigator 3 (*NAV3*), a human analogue to unc-3 (a cell guidance gene of *C. elegans*) belonging to the group of Navigator proteins that bind to the plus-ends of microtubules (+ TIPs, 3), has shown mutations, as well as copy number or expression changes in melanoma, glioblastomas, colorectal cancer, and several other benign and malignant types of tumours (4–14). In addition, NAV3 loss is associated with poor survival in breast cancer and nervous system tumours (15, 16). NAV3 inhibits tumour cell dissemination, most probably by promoting directional migration by stabilizing the microtubules and inhibiting random migration (16). Moreover, NAV3 is induced by tumour suppressor p73 (17), suggesting that it might regulate cancer metastasis.

The ability of tumour cells to modulate extracellular matrix is another prerequisite for invasion and metastasis. Matrix metalloproteinases (MMPs) are a large family of proteases that can degrade all components of extracellular matrix, cleave cell surface receptors, growth factors and other MMPs. Many of the MMPs are overexpressed in melanoma. For example, BRAF mutation leads to MMP9 overexpression, which induces melanoma cell invasion and neoangiogenesis (18). Membrane-bound matrix metalloproteinase 14 (MMP14, MT1-MMP) is often overexpressed in melanoma and regulates a plethora of cellular processes, including cell motility, extracellular matrix mo-

dulation, angiogenesis and metastatic spread (19). MMP2, which is activated by MMP14, also promotes melanoma spreading (20). The clear role of MMPs in tumour progression has led to the development of MMP inhibitors for cancer treatment. Although non-selective MMP inhibitors KDYHIDLOHGLQSKDVH„„VWXGLHVGXHWRODFNRIHIILFDF\ adverse effects, new, more selective, treatment methods, such as MMP-responsive prodrugs, which are activated at the tumour site, are under intense investigation (21).

MMP14 is accumulated at the tips of invadopodia in the invading cancer cells, where it cleaves collagen type I and other matrix molecules enabling cancer cell invasion through interstitial tissues (22). Since microtubules are UHTXLUHGIRU003WUDIILFNLQJWRLQYDGRSRGLDQG NAV3 regulates microtubule formation, the aim of the current study was to explore the possible correlation of NAV3 and MMP14 expression in melanoma.

MATERIALS AND METHODS

Patient samples and melanoma cell lines

The study was approved by the Ethics Committee of Southern)LQODQG 'QU (DQG ($UFKLYDO SDUDIIQ embedded tissue samples of primary melanomas ($n = 27$) and histologically benign naevi ($n = 15$), obtained for diagnostic purposes from patients treated at the Tampere University Central Hospital and Department of Dermatology and Allergology, Helsinki University Central Hospital, Helsinki, Finland, were analysed. In addition, the study examined another 13 melanomas (Breslow WKLFNQHVV 3HUVRU *NAV3*, *MMP14* and *MMP16* mRNA and protein expression. Two established melanoma cell lines, the primary WM-793 (VGP, vertical growth phase) cell line and the lymph node metastasis-derived WM-239 cell line provided by Dr Erkki Hölttä (Biomedicum Helsinki) and originally established by Dr Meenhard Herlyn (The Wistar Institute, Philadelphia, PA, USA (Wistar Institute melanoma cell line collection), were also used.

Probe labelling

Nav3 DNA (RP11-36P3 and RP11-136F16; Research Genetics Inc., Huntsville, AL, USA) and the chromosome 12 centromere probe (pA12H8; American Type Cell Culture (ATCC), Manassas, VA, USA) were ODEHOOHGZLWKGHR[\XULGLQHWULSKRVSKDWH ,Waltham, MA, USA) and Alexa 488-5-dUTP (green; Thermo Fisher 6FLHQWLILFUHVSHFWLYHO[XVLQJQLFNWUDQVODWLRQ7KHSUREHV were mixed with human COT-1 DNA (Invitrogen), precipitated and diluted into hybridization buffer (15% w/v dextran sulphate, 70% formamide in 2× Saline-Sodium citrate (SSC), pH 7.0).

Fluorescence in situ hybridization

(FISH), cell nuclei were extracted, pretreated, and hybridized as described previously (5, 15, 24, 25). FISH signals were analysed both manually (by 2 blinded, independent researchers) and automatically, as described previously (24, 25). The manual analysis was performed using an Olympus BX61 microscope (Tokyo, Japan) equipped with a 60× DQGRLOLPPHUVLRQREMHFWLYHVDQGDWULSOHEDQGSDVVQWHUIRU simultaneous detection of Atto488, Alexa594 and DAPI (Chroma Technology Corp., Brattleboro, VT, USA) according to standard operating procedure (SOP Q2QC005; Dermagene Oy, Helsinki, Finland), which standardizes the signal scoring practice. The results obtained by automat (Metasystems Metafer4 software, Altlussheim, Germany) were checked manually by the 2 of the authors experienced in FISH analysis (PM, SV) and false results were deleted. The result was determined as false, if manually detected signals in the image of a single nucleus did not match with the results provided by the automat. A total of 200–1,000 nuclei were evaluated from each case and the nuclei were grouped as normal if they had 2 signals for chromosome 12 centromere and 2 for the *NAV3*. Relative *NAV3* gain was calculated as the ratio of *NAV3* signals was lower than the number of centromere signals and relative *NAV3* deletion was calculated as the ratio of *NAV3* signals was higher than centromere signals. This method also detects possible aneuploidy (polyploid cells with 3 or more centromere signals). A sample was considered *NAV3* aberrant if the SHUFHQWDJHRIQXFOHLVKRZQJDPSOLFDWLRQGHOHWLRQFHH 7.8%/6.1% cut-off levels (mean ± 2 x standard deviation (2SD)) determined from the benign naevus samples, respectively.

Statistical analysis

Statistical analyses of the FISH results were performed using negaWLYHELQRPLDOUHJUHVVLRQLQ63667KHUHVSRQVHYDULDEOHOL model was the number of nuclei with deletion, and in the second PRGHOWKHQXPEHURIQXFOHLZWKDPSOLFDWLRQ7KHLQGHSHQ variable was the group variable, and the offset variable was the total number of counted nuclei. Multiple comparison of groups was done with Sequential Sidak adjustment method. Kaplan–Meier plot was used when evaluating the survival of the melanoma patients. Patients were divided into groups with relatively more *NAV3* deletions, *NAV3*

Statistical analyses for migration and 3D growth assays were performed with a commercially available statistical software package, IBM SPSS for Mac (IBM, Armonk, NY, USA). For qPCR, the results of *NAV3* correlation with *MMP14* and *MMP16*, Kolmogorov-Smirnov and Shapiro-Wilk tests suggested that both X and Y were non-normal. Therefore, data were log-transformed.

4XDQWLFDWLRQRIP51HSUHVVLRQIRU1$003DQG003

RNA was isolated from 10-µm tissue sections using High Pure FFPE RNA Micro Kit (Roche, Basel, Switzerland) and from WM239 cells using RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was synthesized using SuperScript® VILO™ F'1$VQWKHVLV NLW 7KHUPR)LVKHU 6FLHQWLFF 7KH 7DT0DQ Gene Expression Assay's probe was 20-fold and 0.5 µl was used in 1 reaction. The LightCycler performed 40 runs in each experiment. *NAV3*, *MMP14*, and *MMP16* with iQ Supermix (control 730001045/730003323, cat. 170-8862, (Bio-Rad, Hercules, CA, USA), 1$ Hs00372108_m1; *MMP14*, Hs 01037006_gH; *MMP16*, Hs 00234676_m1, GAPDH, 9)KHUPR)LVKHU6FLHQWLF

Sequence analysis

-bedded tumour samples (QIAamp DNA FFPE Tissue Kit, Qiagen) and *NAV3* primer 5'-ACTGCCACCAGCTCCTTTGGC-3' and reverse primer 5'-TCTCTCAGTTTCGAGGTGGTG-3'). PCR products ZHUHSXULILHG38&3XULILDWLRQ.LW4LDJHQDQG VHTXHQFHG DW Biomedicum Sequencing Unit (Helsinki, Finland). The results were analysed using Gene Composer software (Biomedicum Bioinformatics Unit, Helsinki, Finland).

2-dimensional (2D) wound migration assay

-tion Lab-Tek™ II – Chamber Slide™ System (Thermo Fisher

6FLHQWLILFZDVXVHGIRULQFXEDWLRQDQGJURZWKRI:0HODQ line cultures. When the cell monolayer displayed approximately RYHUFRQXHQFHWKHPRQROD\HUZDVZRXQGHGZWKDVWHULOH rubber blade. The wound closure was assessed 9–24 h later under OLJKWPLFURVFRSHXOOVZLWKHQHGZWKSDUDIRUPDOGHKGH (PFA) in phosphate buffered saline (PBS) for 10 min, washed with PBS, EtOH 35% ethanol (EtOH) in PBS, 50%/PBS and 70%/ PBS, and immersed in 70% EtOH/PBS for storage. The distance migrated by cells was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

51LQWHUIHUHQFH

Small interfering RNAs (siRNA) targeting NAV3 ((FlexiTube GeneSolution for NAV3 GS89795, siNAV3-1 (SI04141956), and siNAV3-2 (SI04272646), Qiagen) and non-silencing control siRNA (SI03650325, Qiagen) were transfected using Lipofecta-PLQH7KHUPR)LVKHU6FLHQWLILF7KHVLOHQFLQJHIFDFLHV were assessed by quantitative polymerase chain reaction (qPCR).

Growth in 3D collagen

Type I collagen (4.8 mg/ml, rat tail, Sigma-Aldrich, St Louis, MO, USA) was mixed with an equal amount of 2 x minimal essential media (MEM), and the pH was adjusted to 7.4 using 20% sodium hydroxide (NaOH). A total of 5,000 cells were suspended in 40 µl hydrogel, the suspension was transferred to a 24-well plate, and incubated for 1 h at 37°C to allow complete gelling. After 48 h incubation in complete growth medium, the cultures were photographed and the percentage of elongated cells amongst the total was calculated using ImageJ software.

Immunostaining

Immunohistochemistry for NAV3 and MMP14 proteins was SHUIRUPHGRQIRUPDOLQHGDQGSDUDIQHPEHGGHGSULPDU\ melanoma samples and 3 metastases, as described previously 6WHU VWDQGDUGHSDUDIQDWLRQWKH VHFWLRQV ZHUHREWHUQHG with ready-to-use 2.5% normal horse blocking serum (Universal Impress, MP-7500, Vector Laboratories, Newark, CA, USA), incubated overnight at 4°C with either rabbit anti-Nav3 (HPA032111, Lot. R32215, Sigma Prestige Antibodies, Sigma-Aldrich) diluted 1:100 in 1% BSA, or mouse anti-MMP14 (MAB3328, Lot. 2450182, Millipore, Burlington, MA, USA), diluted 1:100 in 1% BSA, and further incubated with the secondary antibody Universal ImPress HRP (Universal Impress, MP-7500, Vector Laboratories) and VECTOR NovaRED (SK-4800, Vector Laboratories) substrate. The slides were counterstained with Meyer's haematoxylin and mounted, after graded alcohol series, with Neo-Mount (HX934618, Merck, Rahway, NJ, USA). For the scratch wound slides, immunostaining was performed with the primary antibody anti-Nav3 (HPA032111, Lot. R32215, Sigma Prestige Antibodies, Sigma-Aldrich), diluted 1:300 in BSA 1%, and mouse anti-MMP14 (MAB3328, Lot. 2450182, Millipore) diluted 1:100 in BSA 1%. The slides were incubated overnight at 4°C, followed by the Vectastain imPRESS™ UNIVERSAL REAGENT anti-Mouse/Rabbit Ig peroxidase kit, (Vector Laboratories MP-7500) with Vector AEC Peroxidase Substrate Kit as the chromogen (Vector Laboratories SK-4200) and Mayer's haematoxylin as counterstaining. Slides were mounted with Aquatex (VWR International Ltd, Lutterworth, UK). The immunostainings were analysed independently by 2 authors (OB and AR).

RXEOHLPPXQRÀXRUHVFHQFHVWDLQLQJZKLSDQG003

)RU WKH GRXEOH LPPXQRÀXRUHVFHQFH WKLFN %UHDVOHZ WKLFNQHVVRUPDOOLQHGQGSDUDIQHPEHGGHG

melanoma samples were stained for MMP14 and NAV3. Anti-MMP14 antibody (MAB3328, Lot. 2450182, Millipore) was used at 1:50 dilution in Tris-buffered saline (TBS). The secondary antibody was goat anti-mouse Alexa 594, diluted 1:500 in TBS. The second primary antibody was the same anti-NAV3 antibody as above, used at a dilution of 1:100 in TBS. The primary antibody was incubated overnight at 4°C, and goat anti-rabbit Alexa 488 was used as the secondary antibody, diluted 1:500 in TBS. To visualize the cell nuclei (DNA), the sections were treated with 5RHFKVWVROXWLRQ7KHUPR)LVKHU6FLHQWLILFDQGWKHVOLGHV ZHUHPRXQWHGZWK,PPXPRXQW7KHUPR)LVKHU6FLHQWLILF7KH LPDJHVZHUH YLVXDOLHG XVLQJ LPPXQRÀXRUHVFHQFH =HLVV$,PDJHUPLFURVFRSHRUO=HLVV$2EHUNRFKHQHUPDQ\

RESULTS

1*EHUUDWLRQRIWHRQLRVWSULDUHODQGV *but not in benign naevi*

To determine the possible role of NAV3 in melanoma progression, this study analysed NAV3 (chromosome 12q21) copy number alterations with the use of FISH in primary melanomas and benign naevi. In the primary melanomas, *NAV3* copy number changes were observed in 18/27 (67%) tumours, while no copy number alterations were found in benign naevi (**Table I**). Chromosome 12 polysomy was found in 13/27 (48%) of primary melanomas but not in benign nevi. *NAV3* deletions were found in 16/27 (59%) of the primary melanomas, and, in 5 of these, the proportion of nuclei with deletions was very high, ranging from 46% to 97% (Table I). *NAV3* DPSOLFDWLRQZDVIRXQGLQRIWKHSULPDU\ melanomas, accompanied by chromosome 12 polysomy in 5 cases. *NAV3 DPSOLFDWLRQVZHUH* the tumour cells at most, and 10 tumours (37%) showed heterogeneity in *NAV3* copy number so that both types of aberrated nuclei were present. The proportion of *NAV3* deletions in primary melanomas showed statisti-FDOOVLJQLFDQWGLIIHUHQFHFRPSDUHGZWKEHQLJQQ ($p = 0.02$; Table I). The differences in frequency of *NAV3* DPSOLFDWLRQVGLGQRWUHDFKVWDWLVWLFDOVLJQ

The 2 melanoma cell lines were both heterogenic, since 20% of cells in the primary WM-793 cell culture VKRZHGDPSOLFDWLRQVDQGRIFHOOVVKRZHGGHOHWLRQV of *NAV3*, whereas in the metastasis-derived WM-239 cell FXOWXUH RIFHOOVVKRZHGDPSOLFDWLRQVDQG RI cells showed deletions (Table I).

Since *NAV3* has previously been shown to be mutated in melanoma samples with a nucleotide change c.598C > T

Table I. NAV3 shows copy number changes in cutaneous melanoma samples and in melanoma cell lines

Sample and diagnosis	NAV3 deletion[a]	NAV3 DPSOLFDWLRQ	Chromosome 12 polysomy
Melanoma (n = 27)	16/27 (59%)*	12/27 (44%)	13/27 (48%)
Benign naevus (n = 15)	0/15 (0%)	0/15 (0%)	0/15 (0%)
WM-793 primary melanoma cell line	36 ± 6%	20 ± 9%	83 ± 7%
WM-239 metastatic melanoma cell line	21 ± 7%	9 ± 6%	97 ± 1%

[a]DPSOHZDVIRUGHOHWLRQVLGHUHGWRDEHUUDQWLWKHSHUFHQWDJHRIQXFOHLVKRZLQJDPSOLFDWLRQ deletion exceeded the mean +2 SD determined from the benign naevus samples. The FXWRIIZDVIRUGHOHWLRQDQGIRUDPSOLFDWLRQp = 0.02.

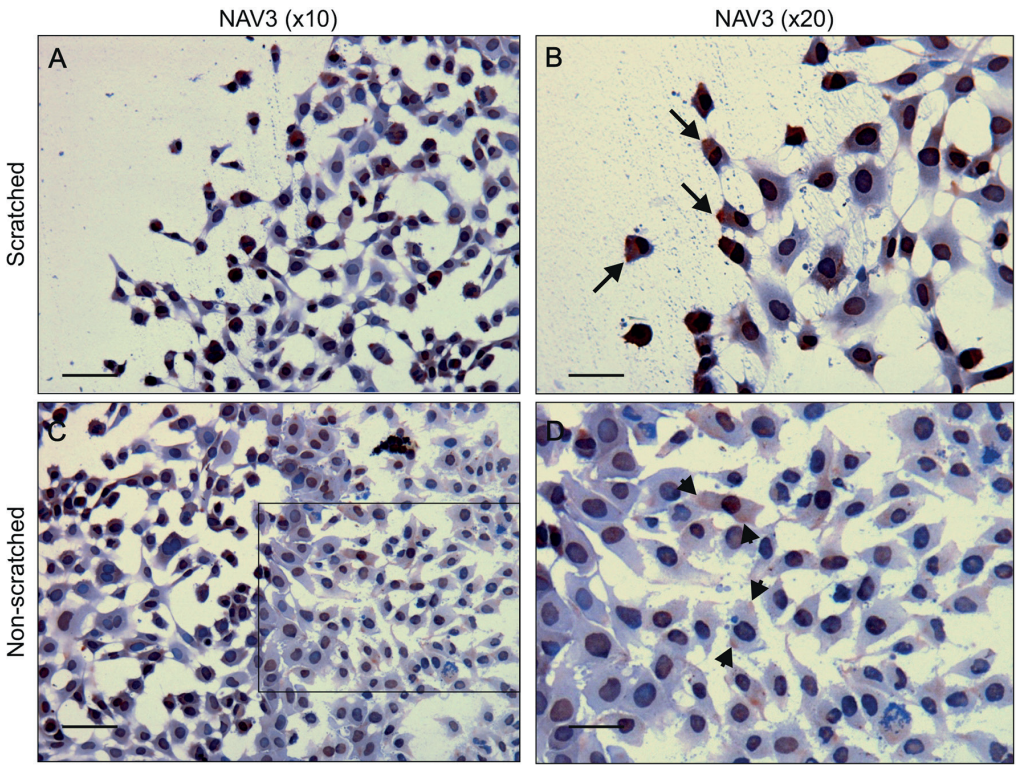

Fig. 1. NAV3 is polarized to the direction of cell movement in WM-239 melanoma cells. (A, B) NAV3 immunohistochemistry (red) of a WM-239 cell culture at the border of a scratched area after 24 h. A unilateral polarization of NAV3 is seen to the direction of cell movement (arrows). (C, D) NAV3 immunohistochemistry of a non-scratched area. NAV3 staining at the cellular membrane is not polarized (arrowheads). (D) Enlargement of the area marked in (C). Scale bar: 100 µm (A, C); 50 µm (B and D).

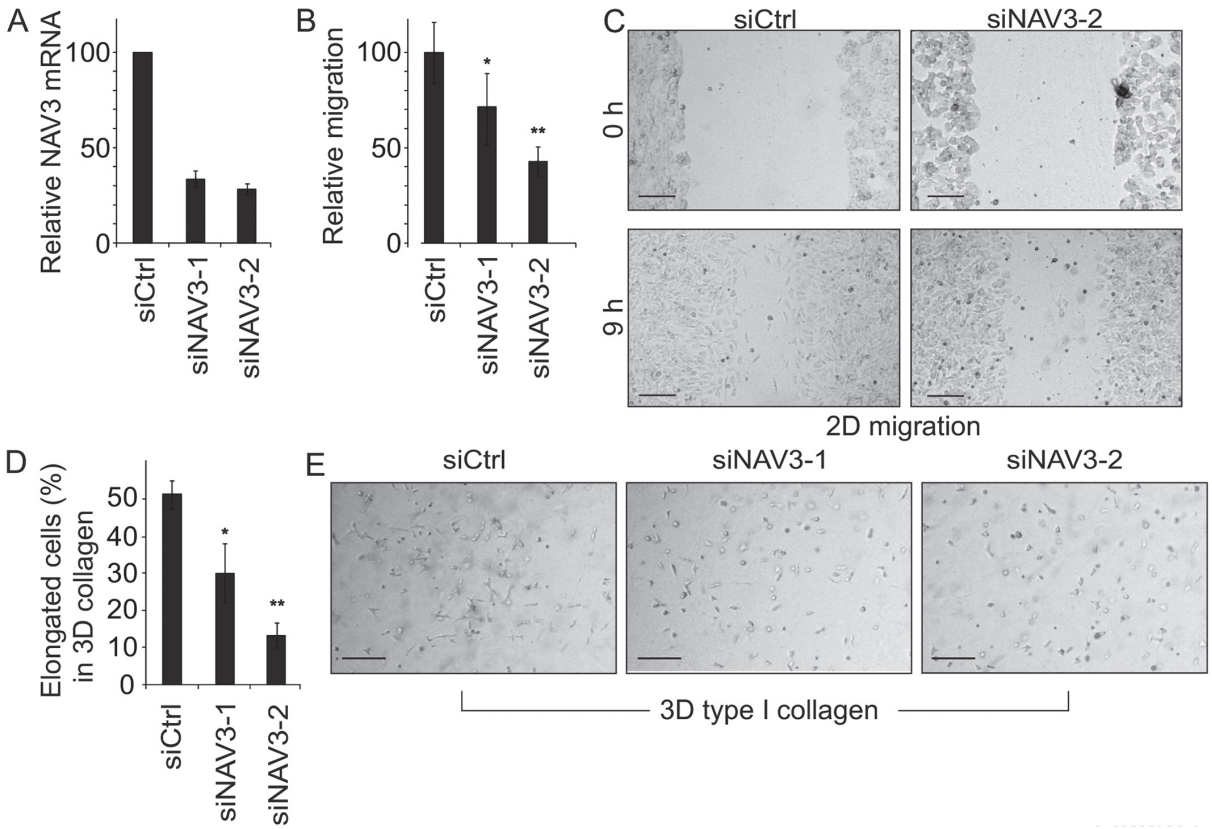

Fig. 2. Silencing NAV3 reduces melanoma cell migration and invasive phenotype in collagen. (A) Relative NAV3 mRNA expression in WM-239 cells transfected with control siRNA (siCtrl) and siRNA targeting NAV3 (siNAV3-1 and siNAV3-2) as assessed by qPCR. (B) Relative distance migrated by WM-239 cells transfected with siCtrl and siNAV3-1 and siNAV3-2 in a 9-h wound scratch migration assay. (C) Light micrographs visualize WM-239 cells transfected with siCtrl and siNAV3-2 at the time points 0 h and 9 h in wound scratch migration assay. Scale bar 200 µm (D) WM-239 cells transfected with siCtrl and siNAV3-1 and siNAV3-2 were grown in 3-dimensional (3D) type I collagen for 48 h. Chart shows the percentage of elongated cells among the total, which indicates the invasive ability of the cells. (E) Light micrographs visualize WM-239 cells grown in 3D collagen type I for 48 h. Scale bar 400 µm *$p < 0.05$, **$p < 0.001$.

(4), the current study analysed the corresponding *NAV3* mutations in the melanoma patient samples (6 patients), but could not detect this transition in any of the samples (data not shown).

1$SURWHLQHSUHVVLRQORFDOLJHVWRWKHHDGLQJHGJH RIPLJUDWLQJPHODQRPDFHOOVLQYLWUR

To understand the role of NAV3 in melanoma cell migration, a 24-h 2-dimensional (2D) scratch wound assay was generated using the WM-239 melanoma cell line. The assay revealed different intracellular localization and patterns of NAV3 staining in the melanoma cells according to the location of the cells, either on the border of the wound or distant from the wound (**Fig. 1**). A striking unilateral polarization of NAV3 was seen at the leading edge of migrating cells (Fig. 1A, B), while no polarization of NAV3 staining was seen in the non-scratched area (Fig. 1C, D). Migrating melanoma cells disclosed the morphology of migrating malignant cells, such as bi- or tri-polar dendritic melanocytes (27).

6LOHQFLQJ RI 1$ UHGXFHV PHODQRPD FHOO PLJUDWLRQ *in 2D environment and invasive growth in 3D type I collagen*

To assess the role of NAV3 in melanoma cell motility, the current study silenced NAV3 from WM239 melanoma FHOOVXVLQJVSHFLILFVL51$ (**Fig. 2**A). In a 9-h 2D wound scratch migration assay, NAV3 silencing with the most HIILFLHQWVL51$ VL1$ UHGXFHGWKHGLVWDQFHPLJUDted by cells by over 50% (Fig. 2B). While control cells exhibited elongated morphology at the migration front, cells silenced for NAV3 remained rounded (Fig. 2C), suggesting that NAV3 is also necessary for microtubule stabilization in melanoma cells.

When the cells were embedded into dense 3D type I collagen, 50% of WM239 cells grown in 3D collagen showed elongated cell morphology and sprouting, UHÀHFWLYHRILQYDVLYHDFWLYLW\LJDQG(1RWDEO\ silencing of NAV3 markedly reduced the cell elongation and invasive phenotype (Fig. 2D and E), suggesting that NAV3 is required for invasive melanoma cell invasive capability inside dense collagen type I.

1$SHSUHVVLRQLQSULPDU\PHODQRPDWXPRXUV

To further investigate NAV3 functions in melanoma, the current study assessed NAV3 protein expression in human melanoma tumours using immunohistochemistry in 39 primary melanomas. NAV3 expression was observed in 27/39 of the melanoma tumours (**Fig. 3**). Notably, NAV3 was strongly and homogenously expressed in all cells of every sample of thin tumours (8/8, Breslow thickness < 1 mm, Fig. 3). In thicker tumours (1–7 mm), in turn, NAV3 was expressed in 19/31 (61%) tumours. In

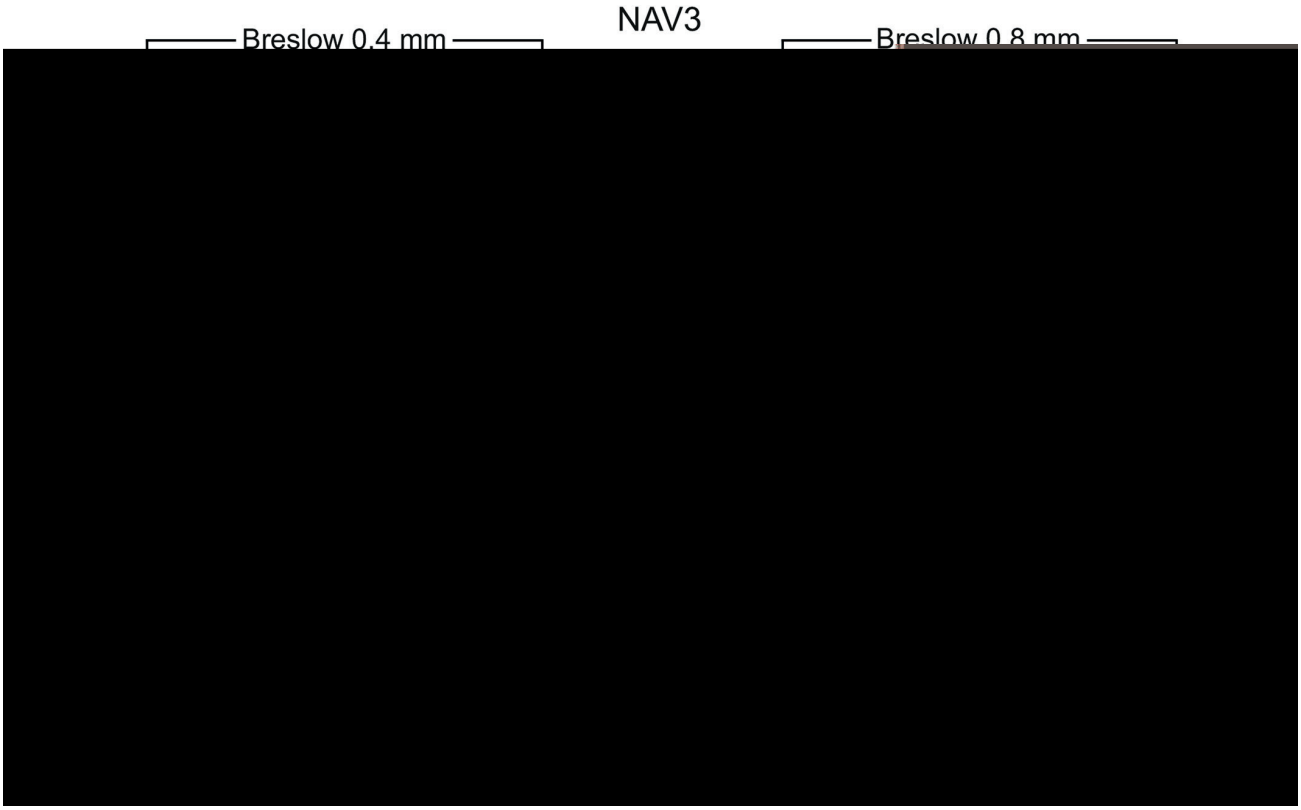

Fig. 3. NAV3 expression is downregulated with increase in melanoma thickness. NAV3 immunohistochemistry of human primary melanoma samples visualizes its expression. NAV3 was expressed in all cells of thin melanomas (Breslow thickness < 1 mm), while in thicker melanomas it was expressed either under the epidermis or at the invasive edge, while in the thickest melanomas (Breslow thickness > 5 mm) it was often lost.